# THE ROLE OF ENERGY METABOLISM IN CUTANEOUS SULFUR MUSTARD INJURY

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## ABSTRACT

Over the past 17 years, our research efforts have focused on systematic studies of the effects of sulfur mustard (2,2'-dichlorodiethyl sulfide, HD) on energy metabolism in human epidermal keratinocytes (HEK). Our objectives are to define mechanisms of HD-induced metabolic injury, determine their role in the cutaneous pathology observed and provide mechanistic information that can be used in development of vesicant agent medical countermeasures. We have characterized effects of HD on the major pathways of cellular energy metabolism in HEK. Our research has shown that inhibition of energy metabolism and depletion of energy stores are a significant consequence of HD exposure and that this inhibition is severe enough to be a determining factor in both cell survival and repair of HD-induced damage. In this paper we present an overview of our results and conclusions to date and briefly discuss their implications.

## 1. INTRODUCTION

Sulfur mustard (2,2'-dichlorodiethyl sulfide, HD) is a vesicating (blister-forming) chemical warfare agent for which there is currently no antidote. It is also a potent alkylating agent, inducing cross-links and strand breaks in DNA, as well as covalent modification of proteins and other cellular components (reviewed in Renshaw, 1946; Papirmeister, et. al.; Sidell, et. al., 1997). As a result, its effects on cells and tissues are varied and complex, and despite years of research, the mechanism(s) by which HD induces blister formation is not understood.

Our current working hypothesis of vesication, which is a modification of that proposed by Papirmeister, et. al. (1985), is that HD penetrates the outer layers of skin to the basal cells at the dermal-epidermal junction resulting in a cascade of biochemical events leading to blister formation. These include: alkylation and damage to DNA; activation of the nuclear enzyme, poly(ADP-ribose) polymerase (PARP); depletion of its substrate, $NAD^+$; inhibition of energy metabolism; protease release and cell death. Most of these components have been demonstrated experimentally and have been well-reviewed over the years (Renshaw, 1946; Papirmeister, et. al.; Sidell, et. al., 1997). However, the details and cause/effect relationships among them, as well as the role(s) of other mechanisms such as apoptosis, necrosis, intracellular signaling, inflammatory mediators, etc., remain under study.

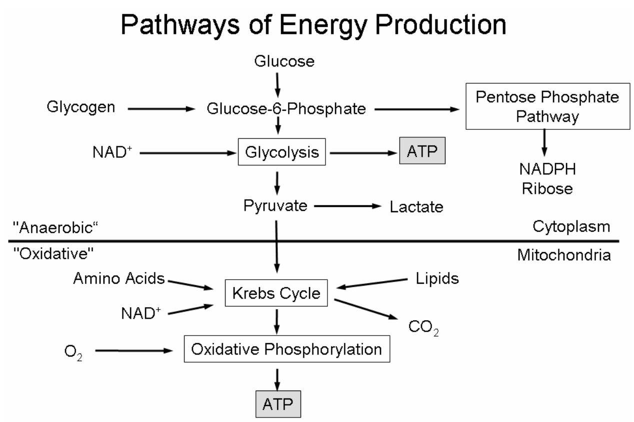


**Figure 1**

The research reported here addresses an important gap in our understanding of the cellular mechanisms of HD injury. Bioenergetic integrity is vital to cell viability and function as well as to the repair of cell/tissue damage. Prior to initiating these studies, however, few metabolic studies had been carried out and most results were pre-1950 (Renshaw, 1946; Papirmeister, et. al.; Sidell, et. al., 1997). In our laboratory, we have spent the last 17 years focused on the effects of HD on energy metabolism in human epidermal keratinocytes (HEK). Our objectives are to define the mechanisms of HD-induced metabolic injury, determine the role of metabolic abnormalities in the observed pathology, and provide mechanistic information that can be used in the development of new vesicant agent medical countermeasures. We have used human basal epidermal cells in culture to (a) characterize the effects of HD on the major pathways of energy metabolism (Figure 1) with respect to the timing, progression and concentration dependence of the effects; (b) correlate them with HD-induced changes in cell viability and overall energy status; and (c) pinpoint specific sites/enzymes targeted by HD. In this paper we

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present an overview of our results and conclusions, to date, and a brief discussion of their implications.

## 2. RESULTS

### 2.1 General Observations

The magnitude of the HD-induced changes that we observed in our experiments depended on the concentration of agent and the time after exposure at which assays were performed. The time-courses, in particular, were characterized by significant quantitative and, in some cases, qualitative differences between "early" (within the first 8 hours) and "late" effects (at 18 to 24 hours).

### 2.2 Effects of HD on Viability & Energy Status

In these experiments (Martens, 1996a; Martens and Smith, 1993), HD at up to 500 µM had no effect on either cell viability (as determined by dye exclusion) or intra-cellular ATP contents within the first 8 hours after exposure. At 18-24 hours we observed concentration-dependent losses in viable cells (cell count · % viability) and intracellular ATP at 100 and 500 µM HD.

Our data are consistent with the findings of Mol et. al. (1989, 1992) in skin and HEK and contrast with those of Meier et. al. (1991). The latter have observed ATP depletion and loss of viability as early as 30 minutes after exposure in peripheral blood lymphocytes, which have been shown to be more sensitive to HD than are HEK (Smith, et. al., 1990). These results suggest that it is the loss of ATP that is the critical final event that precipitates cell death following HD exposure.

### 2.3 Effects of HD on Glycolysis and Intracellular $NAD^+$

The effects of HD on $NAD^+$ and glycolysis were measured in parallel in these experiments (Martens, 1996a) in order to determine the extent of correlation, if any. We observed no adverse effects of HD at ≤ 70 µM. In about 50% of experiments the rates of glycolysis were somewhat higher than those of controls at very early times (≈ 1 hour) after exposure to these low concentrations, suggesting an elevated energy demand for repair of alkylation damage to DNA and/or proteins. Higher concentrations of HD (100 – 500 µM) caused overall inhibition of the rates of glucose utilization and lactate production, depletion of $NAD^+$ similar to that observed by others (Smith, et. al., 1990, 1992; Blank, et. al., 1997), but no change in the levels of $NADP^+$.

When we plotted the rates of glucose utilization and lactate production against intracellular $NAD^+$ at 4, 8 and 24 hours after HD exposure to varying concentrations of HD, we observed linear correlations between the inhibition of glycolysis and depletion of $NAD^+$ at all three time points with slopes as shown in the following table. Although linear, the slopes being less than 1.0 suggests that the inhibition of glycolysis is only partially correlated with $NAD^+$ depletion and that there may be alternative mechanisms involved in the effects of HD on this pathway.

| **Time after Exposure** | **Slope (Rate vs. $NAD^+$ Content)** | |
|---|---|---|
| | **Glucose Utilization** | **Lactate Production** |
| 4 & 8 hours | 0.6 - 0.7 | 0.6 - 0.7 |
| 24 hours | 0.9 - 1.1 | 0.5 - 0.7 |

In control HEK, both glucose utilization & lactate production were linear with time and resulted in a ratio of lactate produced/glucose used of 1.9 ± 0.1, which is statistically identical to the theoretical ratio of 2.0 expected for complete conversion of glucose to lactate via anaerobic glycolysis. In exposed cells the ratio did not differ from controls in the first 8 hours after exposure to 100 – 670 µM HD. We conclude from this data that glucose metabolism in control and "early" HD-exposed HEK is primarily anaerobic, with negligible contributions from oxidative metabolism or the pentose phosphate pathway. At 18-24 hours after exposure, however, glucose utilization was more severely inhibited by HD than lactate production. This resulted in lactate/glucose ratios that increased linearly with HD concentration up to a maximum of ratio of 5, indicating a shift in metabolism toward the production of excess lactate.

We propose three possible explanations for the increase in the lactate/glucose ratio that we have observed. They are: (a) that inhibition of mitochondrial metabolism may cause accumulation of amino acids and/or Krebs cycle intermediates, which are then converted to pyruvate and excreted from the cell as lactate, (b) that an increase in the ratio of $NADH/NAD^+$ due to PARP activation may cause an imbalance in the redox potential of the cell that drives the conversion of pyruvate to lactate, and/or (c) that extra riboses resulting from degradation of poly(ADP-ribose) in the nucleus may be catabolized to lactate after conversion to hexoses and trioses in the non-oxidative branch of the pentose phosphate pathway.

### 2.4 Effects of HD on Pentose Phosphate Pathway

The activity of the oxidative branch of the pentose phosphate pathway was determined by measurement of the rate of $^{14}CO_2$ production from 1-$^{14}$C-glucose (corrected for $^{14}CO_2$ production from 6-$^{14}$C-glucose via the Krebs cycle) (Martens, 1998). The activity in untreated cells comprised only 2 - 3% of the total glucose consumption. At 300 µM HD, we observed a 40% inhibition of the overall activity measured over 24 hours (vs. 65% inhibition of glycolysis). No effect was seen in 0 to 3-hour experiments (vs. 35% inhibition of glycolysis) or at concentrations of HD less than 300 µM.

These data suggest three possible scenarios: (a) glycolysis and the pentose phosphate pathway are inhibited by different mechanisms or at different sites, (b) inhibition is at a common site, with the relative degree of inhibition depending on the extent of control this site exerts on the flux through each pathway, or (c) equal inhibition at a common site, but the inhibition of the pentose phosphate pathway is cancelled out by activation at a site distal to that of inhibition.

### 2.5 Effects of NAD$^+$ Depletion

***Effects of Niacinamide:*** Niacinamide, which is both a PARP inhibitor and a precursor for the synthesis of NAD+, has been shown to protect HEK against HD-induced NAD+ depletion (Smith, et. al. 1992). In order to test the hypothesis that NAD+ depletion is the cause of HD-induced inhibition of glycolysis, we pretreated HEK with varying concentrations of niacinamide prior to exposure and then assayed for both NAD+ content and the rate of glycolysis. In these experiments (Martens and Smith, 1993), 1 mM niacinamide increased NAD+ levels at all time points in both control and exposed cells. No effects were seen at less than 1 mM. At 100 µM HD, NAD+ depletion was completely prevented (i.e. maintained at naïve control levels or higher) for up to 24 hrs. At 500 µM HD, we observed complete protection at 4 hours, partial protection at 8 hours (75% of naïve control) & 24 hours (55% of naïve control).

Despite the ability of niacinamide to preserve NAD$^+$ in these cells, we observed no protection against the loss of viable cells (cell count · % viability), inhibition of glucose metabolism or depletion of ATP. These data are consistent with the findings of Mol et. al. (1991) in skin and HEK. In contrast to our data, however, Meier (Meier, et. al., 2000; Petrali, et. al, 1990) have reported that in peripheral blood lymphocytes, niacinamide protects against the loss of cell viability, NAD$^+$ and ATP. This suggests that at least some of the biochemical changes induced by HD may be cell-type specific.

We conclude that HD-induced alterations in glucose metabolism involve complex metabolic changes that depend on the timing and/or degree of injury. NAD$^+$ depletion does not cause inhibition of glycolysis or depletion of ATP. Rather, we suggest that HD inhibits glycolysis directly, at the level of catalysis and/or regulation of one or more enzymes and that the site of inhibition lies at the beginning of the pathway, perhaps at the level of the hexokinase reaction.

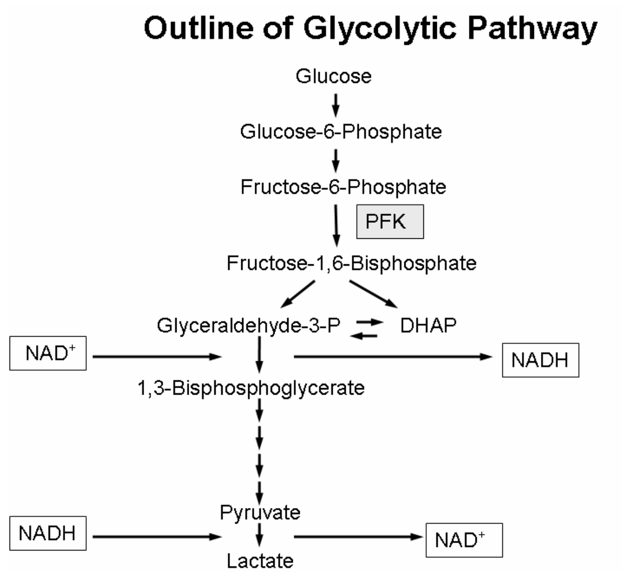


**Figure 2**

***In silico Model of Glycolysis:*** To test hypotheses and predict potential sites of inhibition by HD, we developed a computer model of glycolysis using literature values for the rate equations and mass-action ratios (Martens and Sweeney, 1998). We have used this model to test the hypotheses that HD-induced inhibition of glycolysis is caused by depletion of NAD+. NAD+ was removed from the model using a first-order rate constant chosen to cause the decrease in NAD+ to follow an experimentally determined time-course (Martens, 1996a). The results showed transient inhibition of (a) flux through the enzymes downstream from phosphofructokinase (PFK) (see Figure 2) and (b) the overall rate of lactate production. There was no change in the flux through PFK or enzymes upstream therefrom or in the overall rate of glucose utilization. The only significant, long-term change was an increase in the NADH / NAD+ ratio.

***Intracellular Redox State:*** Preliminary studies of HD effects on intracellular redox state (unpublished data) have shown that HD appears to cause a change in the redox state of the cell such that the cytoplasm becomes more reduced (measured with fluorescent probe). We speculate that the causes of this phenomenon are (a) activation of PARP, which causes depletion of NAD+ but not NADH and/or (b) inhibition of mitochondrial electron transport, which can cause build-up of reducing equivalents in the cell. Such a change could potentially cause additional adverse effects on metabolism that are secondary to the initial insult, as several key metabolic enzymes are regulated by the NAD(P)H / NAD(P)+ ratio.

### 2.6 Effects of HD on Oxidative Metabolism

***Glucose Metabolism:*** Oxidative metabolism of glucose via the Krebs cycle was determined as the production of 14CO2 from 6-14C-glucose (Martens, 1998). The observed rates were less than 0.1% of the total rate of glucose utilization, which was close to the limit of detection for the assay. Exposure to 300 µM HD appeared to have no effect on the rate. Our data show glucose metabolism in HEK is primarily anaerobic, with negligible rates of oxidative metabolism. This is consistent with what has been observed for skin metabolism *in vivo* (Decker, 1971).

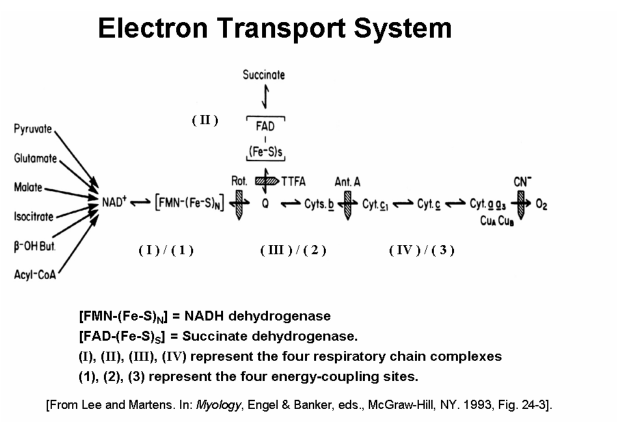


**Figure 3**

***Substrates linked to Krebs cycle:*** Despite the lack of oxidative glucose metabolism, HEK still require the ability to metabolize amino acids, lipids and other small molecules that feed into the Krebs cycle. We have examined the effects of HD on the oxidation of glutamine, palmitic acid and the Krebs cycle intermediate, succinate (Martens, 1996b, 2002, 2006). In all cases we observed both time- and concentration-dependent changes as well as significant differences between "early" vs. "late" effects of HD.

Within the first 4 hours after exposure, 100 – 500 µM HD caused inhibition of glutamine, palmitic acid and succinate oxidation. Succinate oxidation was less sensitive to inhibition than was oxidation of the NAD-linked substrates. At 18 - 24 hours after exposure, 300 – 500 µM HD caused severe inhibition of the oxidation of glutamine and palmitate, but not of succinate.

Glutamine and palmitic acid oxidation are linked to the electron transport chain via reduction of $NAD^+$ to NADH, whereas succinate oxidation is linked through reduction of the flavin FAD (Figure 3). Our data suggest (a) that one or more enzymes comprising respiratory complex I may be directly inhibited by HD, (b) that complexes II, III and IV are relatively insensitive to HD, and (c) that the severe respiratory inhibition seen at 18 – 24 hours may result from mitochondrial membrane damage, collapse of pH gradient ($\Delta pH + \Delta\Psi$) and loss of energy transfer capability.

***Mitochondrial Membrane Integrity:*** We have determined the effects of HD exposure on mitochondrial membrane permeability by fluorescence microscopy using the mitochondrial membrane potential probe JC-1 (unpublished data). We observed no changes either at ≤ 8 hours with up to 500 µM HD or at 18-24 hours with 100 µM HD. However at 24 hours, 300 or 500 µM HD caused a loss in the mitochondrial membrane potential ($\Delta pH + \Delta\Psi$) that paralleled the loss of cell viability & observable changes in cell structure.

***Causes of Metabolic Cell Death:*** We have also carried out experiments (unpublished data) to determine which of the energy-producing pathways are likely to be the most critical determinants of HD-induced cell death in HEK. Our results show that, in the absence of HD, compounds which inhibit either glycolysis (e.g. 2-deoxyglucose) or mitochondrial electron transport (e.g. rotenone + antimycin) alone do not kill HEK. In order to induce cell death, treatment with inhibitors of both glycolysis and electron transport was required. Alternatively, we could also induce cell death with compounds that collapse the mitochondrial pH gradient (e.g. uncoupling agents) in the absence of other inhibitors.

In summary, we observe changes in mitochondrial energy metabolism that correspond temporally with both ATP depletion and loss of cell viability. We conclude that mitochondrial injury in combination with glycolytic inhibition can push HEK into metabolic crisis and cell death and that protection of the mitochondrial membrane potential is critical for cell survival after HD exposure.

## CONCLUSIONS

In summary, our data show that HD inhibits both anaerobic and aerobic metabolism in HEK, causes a shift in metabolism toward the production of excess lactate, and alters the intracellular redox state. The metabolic inhibition occurs many hours before ATP loss and the onset of cell death, which suggests that these pathways may provide potential targets for early therapeutic intervention. We found little or no evidence to support the theory that HD-induced $NAD^+$ depletion is the cause of inhibition. Rather, we suggest that HD inhibits one or more enzymes of glycolysis at the level of catalysis and/or regulation and that the site of inhibition is likely to lie at the beginning of the pathway. In the later stages of injury, we observed collapse of the mitochondrial membrane potential, depletion of ATP and loss of cell viability -- hallmarks of the final stages in irreversible cellular damage.

Our research shows that inhibition of energy metabolism and depletion of energy stores are a significant consequence of HD exposure. We propose that the combination of HD-induced inhibition of mitochondrial and glycolytic pathways pushes HEK into metabolic crisis leading to cell death, that alterations in intracellular redox potential may cause even further metabolic injury, and that protection of mitochondrial membrane potential is critical for cell survival. The metabolic injury we have observed is severe enough to cause pathology, inhibit DNA replication and protein synthesis, lessen ability to repair cellular damage, and impair wound-healing in the skin.

In recent years, there has been increasing evidence for the importance of mitochondrial injury in both apoptosis and necrosis (Mignotte and Vayssiere, 1998; Skulachev, 2006). There has also been an upsurge in clinical research into understanding and treating diseases of energy metabolism that has led to some innovative and successful treatments (Zorov, 1996; Kiningham, et. al., 1999; de Grey, 2000). Similar strategies may be found to be effective against HD-induced metabolic injury.

The results of our research help to fill an important gap in our understanding and add to the growing knowledge base of cellular mechanisms in cutaneous HD injury. Strategies to preserve the bioenergetic integrity of cells and tissues may become key components in our arsenal of vesicant agent countermeasures.

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